Navigating the Vast Array of Sample Preparation Techniques for Biological Samples – Whole Blood

by Matt Brusius, Phenomenex, Torrance, CA USA

Work place drug and pain management testing has historically been performed in urine samples. However, urine is typically more indicative of what was previously introduced into the body as opposed to the more complete and real time indicator that whole blood testing offers which explains its popularity in the world of Forensic Toxicology. Unfortunately, whole blood is significantly more complex and requires thorough sample preparation even prior to ultra-selective cleanup techniques like solid phase extraction (SPE), let alone before analysis by GC-MS/MS or LC-MS/MS.

The appropriate pretreatment of whole blood prior to SPE is vital for accurate quantification. Two challenges that must be overcome are ensuring complete disruption of analyte protein interactions (not normally found in urine) and release into the liquid portion of the blood. This pretreatment usually involves a haemolysis step to release the drugs that may have been taken up by the erythrocytes followed by a subsequent protein precipitation, or other form of sample preparation, which must ensure that analytes do not co-precipitate out of solution. Implementing such a pretreatment for a wide range of chemically diverse analytes can prove challenging as their intrinsic physiochemical properties have a significant impact on what solvents they can be extracted into.

Determining the Most Effective Pretreatment Step

To determine the most effective approach to whole blood testing, an experiment was designed that evaluated different pretreatment options to prepare whole blood for an SPE method with the goal of determining which option(s) resulted in the highest recovery for each analyte class. Since the effectiveness of the sample preparation is determined in part by the chemical properties of the analytes, testing a wide range of forensically relevant compounds (Table 1) is necessary, and there may not be one pretreatment that is best across all classes of compounds. The scope of this study includes mostly basic compounds with the exception of some neutrals like carisoprodol and some benzodiazepines. These compounds range from moderately hydrophobic (methadone, Log P = 5.01) [4] to relatively polar (benzoylecognine, Log P = -0.59) [4]. However, overall Log D will be more effective at predicting solubility and consequently the recovery for a particular precipitating solvent.

In this method, whole blood pretreatments are broken down into two important steps: haemolysis and protein precipitation (followed by centrifugation).

Haemolysis – osmotic pressure and metal induced protein denaturation

Osmotic breakdown:

In order to reproducibly quantify the *total* drug and metabolites present it in a blood sample, it is necessary to lyse the red blood cells to account for any drug taken up by the erythrocytes in addition to the surrounding plasma.

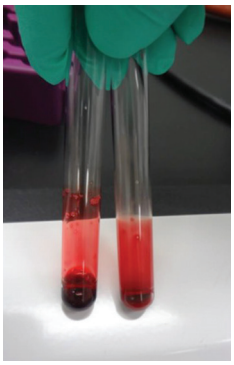

Figure 1: Lysed Blood (left) vs Unprepped blood (right) [1].

One of the most popular lysing approaches is done with water, often times referred to as osmotic breakdown. The method is simple and requires a 1:1 dilution of the whole blood with water followed by simple shaking (vortex or sonication is preferred). Figure 1 shows a side-by-side comparison of an unprepared blood sample and one that has been lysed via osmotic breakdown. On the left, the whole blood sample that has been lysed is easily distinguished because the lysed blood cells do not stick to the side of the culture tube.

Inorganic denaturing:

The second pretreatment evaluated was the use of zinc sulphate ($ZnSO_4$) as a protein denaturant where 100 µL of 5% $ZnSO_4$ was added to 500 µL of whole blood. Zn^{2+} binds to proteins in the blood, forming insoluble metal-protein salt complexes which cause the membrane proteins of the erythrocytes to precipitate, lysing the cell. In addition, as Zn^{2+} binds to the coordinated amino acids, protons are displaced, thus decreasing the pH of the sample [5], which can decrease Log D values and improve solubility in acetonitrile. As the red blood cells lyse, the haemoglobin enters the liquid part of the blood causing the bright colour change providing visual confirmation.

Comparing the two methods:

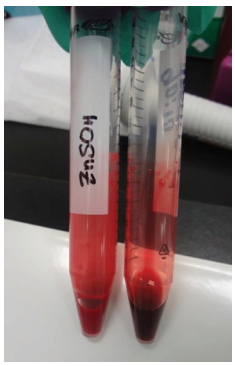

Figure 2: $ZnSO_4$ Precipitation vs Osmotic Breakdown [1].

Figure 2 shows the difference between the $ZnSO_4$ haemolysis and the osmotic breakdown product. $ZnSO_4$ appears to be more effective at lysing the red blood cells and thus produces a much brighter red solution, indicating a more effective lysing step [1].

Precipitation

For the scope of this investigation, protein precipitation by acid and by water miscible organic solvents are explored, both of which rely on different chemical principles. Organic solvents cause precipitation of proteins by significantly lowering the dielectric constant of the whole blood solution, which causes the electrostatic interactions between proteins to increase. The solvent also removes any of the surrounding water shell that effectively minimises the hydrophobic interaction between proteins, thus causing electrostatic interactions to become paramount which leads to protein aggregation [5].

Acid precipitation operates by a somewhat similar mechanism in the sense that it helps pull ordered water away from the surface of the protein; however, in this case it is due to hydration of the salts present in solution. The end result of this phenomenon is by contrast an enhancement of hydrophobic interactions between proteins which causes aggregation [5].

Acid precipitation:

Acid precipitation is well characterised and was initially intriguing because of the variety of acids that can be used to facilitate this process. It is also commonly used as a modifier in acetonitrile organic precipitations that function to protonate the carboxyl groups of the proteins, thus excluding acetonitrile which increases the solubilising effect for nonpolar groups leading to improved recovery [7]. The options considered in this work are 10% TCA and 6% $HClO_4$. In both cases 250 µL of the acid was added to 500 µL of whole blood diluted with 500 µL of water.

In both of these cases (Figures 3 and 4), the supernatant that was produced was clear but also contained deposits and other cellular materials that stuck to the sides of the tube post centrifugation. The perchloric acid being a stronger 'super acid' resulted in more pronounced cellular deposits.

Figure 3: TCA precipitation post centrifugation (left) and Figure 4: $HCl0_4$ post centrifugation (right) [1].

Water miscible organic precipitation:

Mixtures of acetonitrile and methanol – a tradeoff between efficient precipitation, and analyte solubility.

It has been shown that acetonitrile does a better job of precipitating proteins out of solution than methanol [5]. This is most likely due to acetonitrile's ability to more efficiently remove ordered water as its triple bond pi-stacks with cationic and aromatic moieties on the protein's surface [6]. In addition, as an aprotic solvent it will readily accept and hydrogen bond with free waters. However, as it is noted previously, adding acid to an acetonitrile precipitation can improve recovery of compounds [7] and methanol's role as a protic solvent most likely contributes to the same effect, helping to improve the solubility of analytes of interest and boost recovery of hydrophobic compounds. It is for that reason that 10:90 (v/v), 50:50 (v/v) and 90:10(v/v) ACN:MeOH were investigated.

After the osmotic breakdown was performed by using a 1:1 dilution with water and light vortexing, a 3:2 ratio of organic to sample was tested while varying the ratio of acetonitrile to methanol as precipitating reagents which has been previously shown to effectively remove proteins from human plasma samples [5]. Figure 5 shows that the increased amount of acetonitrile, 90:10 (v/v) ACN:MeOH yields a brighter red colour in comparison to the methanol precipitate. This suggests that acetonitrile does a better job releasing haemoglobin and potentially any drug bound analytes into the liquid part of the sample. This is supported by previous work that states that haemolytic and non-haemolytic samples can be easily differentiated by their colour but should be confirmed via UV [8]. However, even complete haemolysis does not necessarily ensure that the analytes are totally broken up from the crashed out proteins, which can impact recovery.

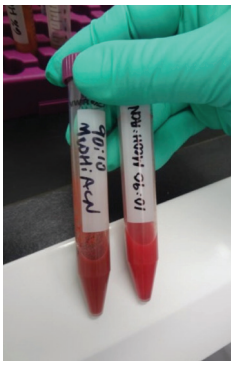

Figure 5: ACN:MeOH (90:10) v/v vs. ACN:MeOH (10:90) v/v precipitation of whole blood [1].

In addition to producing a brighter red colour, the 90:10 (v/v) ACN:MeOH precipitate also forms a clearer supernatant as seen in Figure 6.

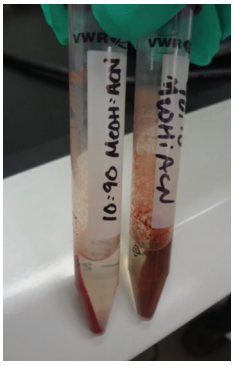

Figure 6: 90:10 (v/v) ACN/ MeOH precipitation of whole blood [1].

$ZnSO_4$ + organic solvent:

The use of an inorganic salt such as $ZnSO_4$ and organic precipitating reagents were also evaluated. As seen in Figure 7, these results follow the trend described in the previous section (organic precipitation), where a higher concentration of acetonitrile yielded brighter red samples, and correspondingly clearer supernatant Figure 8.

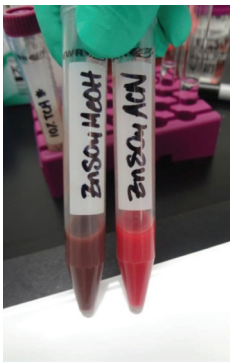

Figure 7: $ZnSO_4$ MeOH (left) vs. $ZnSO_4$ ACN (right) precipitation of whole blood [1].

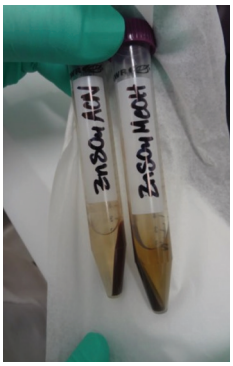

Figure 8. $ZnSO^{4+}$ACN (left) vs $ZnSO_4$ (right) precipitation of whole blood [1].

Post Dilution Comparison

To prepare the sample for SPE, pretreatments that influence cation-exchange and reversed phase SPE cleanup had to be considered. Acidifying the sample with 0.1% formic acid ensures that all bases are ionised and able to interact with the sulphonic acid cation-exchange moiety. The aqueous dilution also serves a second purpose which is to dilute the organic solvent making it more amenable for reversed phase interaction. However, it serves neither of these purposes in the context of the acid precipitation as the sample is already acidic and 100% aqueous.

Both the organic precipitating reagents with osmotic breakdown and the $ZnSO_4$ lysed cells with acetonitrile showed some degree of turbidity after dilution (Figures 9 and 10), which may imply that further cleanup is necessary. This most likely occurred for one of two reasons, the formic acid caused further protein precipitation in the

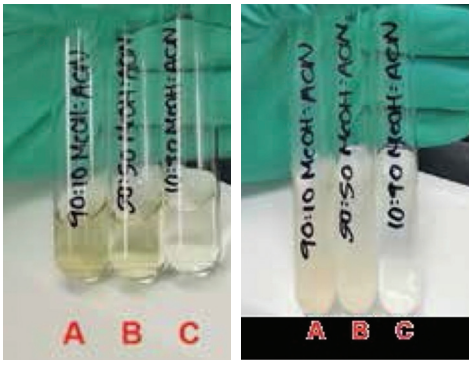

Figure 9: ACN:MeOH precipitates pre and post dilution [1].

sample resulting in the cloudy sample and/or the water addition caused many of the particulates (and possibly analytes) to fall out of the previously all organic solution.

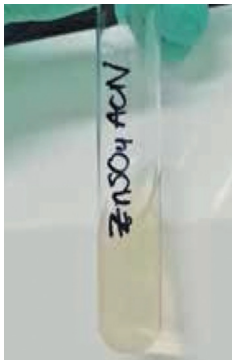

Figure 10. $ZnSO_4$ ACN precipitate post dilution [1].

Based on the lack of turbidity observed with the acidic precipitated samples post dilution (Figure 11), it could suggest that formic acid is at least partially responsible for the cloudiness. However, it is also possible that the same matrix components that caused the turbidity in the organic precipitates were not present in the acidified precipitation since they may not have been miscible in the acidic solution and thus were never there to be crashed out in the first place.

Solid phase extraction

Figure 11: 6% Perchloric acid precipitate post dilution (left) and 10% Trichloroacetic acid precipitate post dilution (right) [1].

Table 1 summarises the suite of compounds employed in this study that range from polar bases (opiates) to hydrophobic neutrals (benzodiazepines). Because of the neutral and basic drugs in this panel, a polymeric mixed mode cation-exchange SPE cartridge (Strata-X-C, Phenomenex) was chosen to utilise both its hydrophobic retention as well as its cation-exchange capability. The SPE protocol and pretreatment use a 0.1% formic acid wash followed by a 30% organic wash. The formic acid wash keeps analytes protonated and helps remove any lightly bound polar and acid interferences while the 30% methanol wash is strong enough to remove any lightly bound moderately hydrophobic interferences, but not too strong such that it would compromise the recovery of non-ionic bases such as the benzodiazepines. The elution scheme of Ethyl Acetate:IPA:Ammonium hydroxide (7:2:1) v/v work together to disrupt hydrophobic, polar and ionic interactions ensuring complete recovery is achieved by the elution solvent. These are optimised elution conditions as this percentage of ethyl acetate is strong enough to dislodge hydrophobic analytes, but is not strong enough to elute contaminants such as phospholipids and fatty acids that are present from the matrix. The final elution solvents for each pretreatment step are visually compared in Figure 12.

Class	Analyte	Class	Analyte
Benzodiazepines	Alprazolam	Synthetic Opioids	Methadone
	Clonazepam		EDDP
	Diazepam		Fentanyl
	Flunitrazepam		Norfentanyl
	Lorazepam		Meperidine
	Midazolam		Normeperidine
	Nordiazepam		Naloxone
	Oxazepam		Norpropoxyphene
	Temazepam		Propoxyphene
	α-Hydroxyalprazolam		Sufentanil
	Alprazolam		Naltrexone
Opiates	Codeine	Amphetamines	Amphetamine
	Hydrocodone		Methamphetamine
	Hydromorphone		MDMA
	Morphine		MDA
	6-Acetylmorphine (6-MAM)		MDEA
	Oxymorphone	Analgesics	Tramadol
Illicit Drugs	Phencyclidine		Carisoprodol
	Benzoylecgonine		Buprenorphine
			Norbuprenorphine

Table 1. Compounds in pain panel [1].

SPE Procedure:

Pretreatment: Add 100 µL 5% (w/v) ZnSO4 to 0.5 mL whole blood (with EDTA preservative) in a glass tube and vortex for 3-5 seconds. Add 1.5 mL chilled (~0°C) 90:10 ACN/MeOH while vortexing. Centrifuge the samples at 6,000 rpm for 10 minutes and transfer the supernatant to a new glass tube. Add 4 mL of aqueous 0.1% formic acid to the supernatant to acidify and dilute the mixture. The sample is now ready for SPE (using a Strata-X-C 30mg/3mL SPE cartridge).

Condition: 1 mL Methanol
Equilibrate: 1 mL Water
Wash 1: 1 mL 0.1% Formic acid in water
Wash 2: 1 mL 30% Methanol in water
Dry: 3 to 4 minutes at high vacuum (~10" Hg)
Elute: 2x 500 µL (2 aliquots) of 500 µL) Ethyl acetate:Isopropanol:Ammonium hydroxide (7:2:1)
Dry Down: Evaporate to dryness under nitrogen at 40-45°C
Reconstitute: With 500 µL of 85:15 (A:B) of LC mobile phase

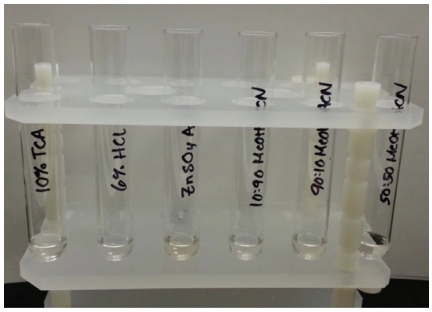

Figure 12: Final elution for each pretreatment option [1].

LC/MS/MS

This method was run using a Kinetex 2.6 µm core-shell Biphenyl HPLC/UHPLC column (Phenomenex). This superficially porous column offers improved efficiency in comparison to fully porous columns and the selectivity of the biphenyl ring structure is a great choice for pain panel methods that include opiates, benzodiazepines and other drugs containing aromatic moieties. The resulting LC/MS/MS method is outlined below and the resulting chromatogram is depicted in Figure 13.

LC/MS/MS Method

Dimensions: 50 x 3.0 mm

Mobile Phase: A: 0.1% Formic acid in water
B: 0.1% Formic acid in methanol

Flow Rate: 0.7 mL/min

Gradient:

Time (min)	% B
0.00	100
2.50	100
3.50	100
3.51	10
5.00	10

Temperature: Ambient

Detection: MS/MS, ESI+
(4000 QTRAP®, SCIEX)

Injection: 10 µL

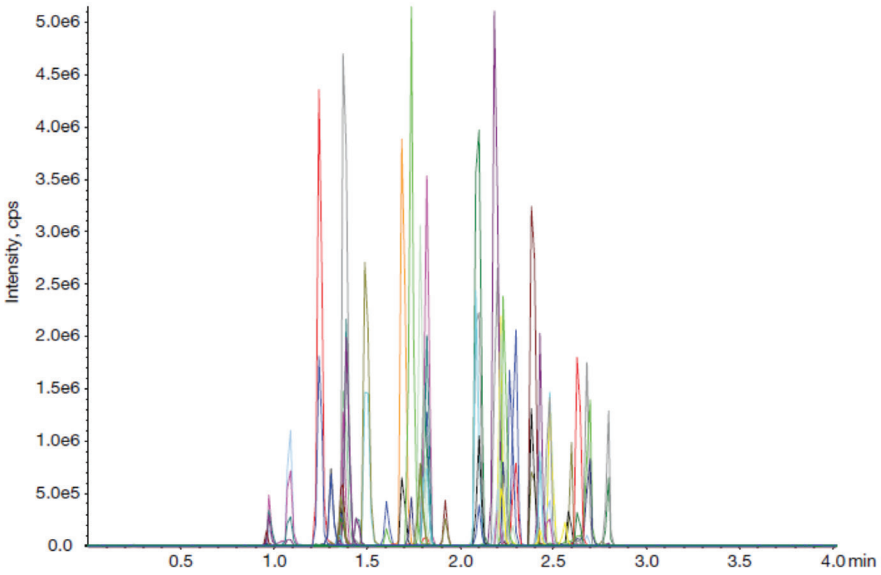

Figure 13: Representative Chromatogram of Basic Compounds [1].

Comparison of Pretreatments:

Acidic Pretreatments

While the acidic pretreatments of $HClO_4$ and TCA produced the clearest supernatants upon aqueous dilution (Figure 11), they also produced the poorest recovery (Figures 14, 15 and 16). This could possibly be explained by solubility or stability issues. Since the majority of the compounds in this suite are hydrophobic and basic it is plausible to suspect that the analytes themselves were not miscible in the very polar acidic solution which forced co-precipitation along with the proteins.

While acidic precipitation yielded generally the lowest recoveries, it is important to note that the recovery of some compounds were close to the optimal precipitating solvent. The compounds

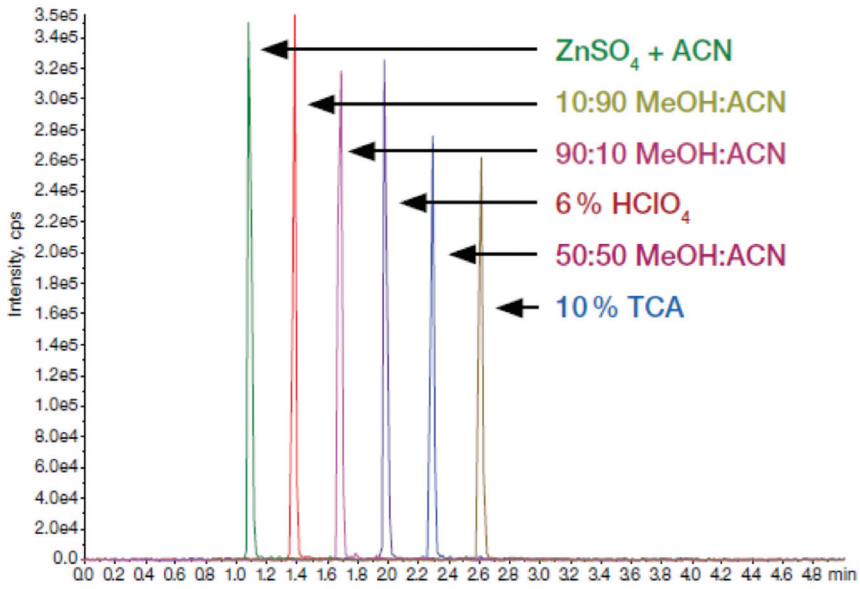

Figure 14: Comparison of the effects of various pretreatment options on amphetamine. Chromatograms are overlaid with time shift to provide clarity [1].

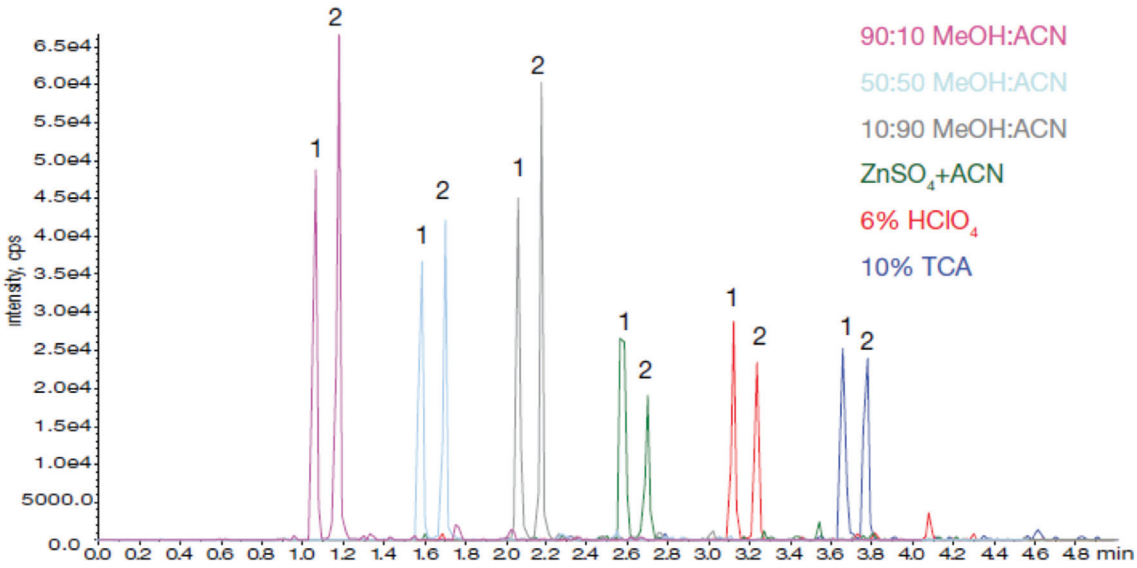

Figure 15: Comparison of the effects of various pretreatment options on Codeine (peak 1) and Hydrocodone (peak 2) Chromatograms are overlaid with time shift to provide clarity [1].

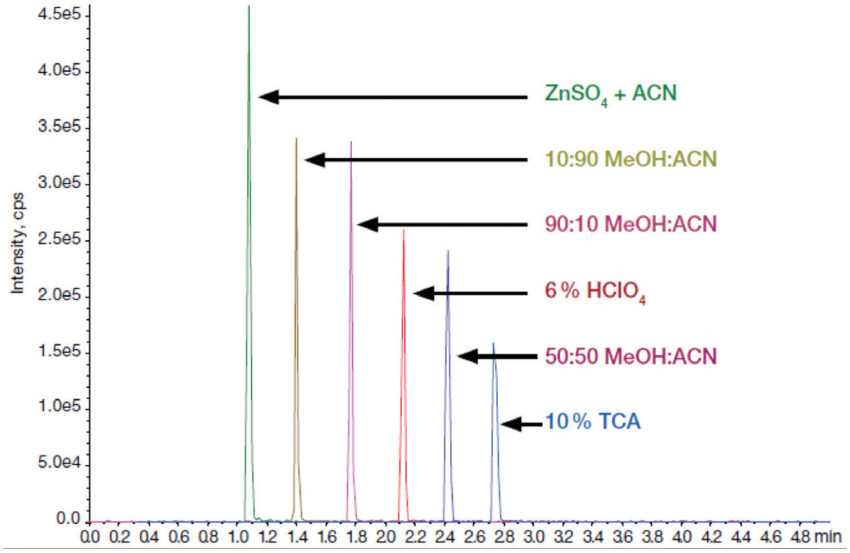

Figure 16: Comparison of the effects of various pretreatment options on Benzoylecgonine. Chromatograms are overlaid with time shift to provide clarity [1].

Analyte	Log P	pKa	Log D at pH 7	Log D at pH 1
Alprazolam	3.02	5	3.02	-1.81
Clonazepam	3.15	2	3.15	1.17
Diazepam	3.08	3	3.08	0.3
Flunitrazepam	2.55	2	2.55	0.7
Lorazepam	3.53	NA	3.69	3.69
Midazolam	3.97	4	3.97	0.4
Nordiazepam	3.21	2.85	3.21	0.48
Oxazepam	2.92	NA	2.92	2.92
Temazepam	2.79	NA	2.79	2.79
Codeine	1.34	9	-0.52	-1.84
Hydrocodone	1.96	9	0.12	-1.2
Hydromorphone	1.62	9	-0.11	-1.35
Morphine	0.9	9	-1	-2
6-MAM	1.09	8.4	-1.18	-2.44
Oxymorphone	0.78	8.2	-1	-2.2
Phencyclidine	4.49	11	1.35	1.35
Benzoylecgonine	-0.59	9.5	0.2	-0.9
Methadone	5.01	9	2.55	1.89
EDDP	4.63	9.5	2.18	1.5
Fentanyl	3.82	9	1.3	0.65
Norfentanyl	1.42	10	-1	-1.49
Meperidine	2.46	8	1.32	-0.66
Naloxone	1.62	8	0.43	-1.5
Norpropoxyphene	4.52	10	2.1	1.74
Propoxyphene	4.9	9	2.5	1.8
Sufentanil	3.61	9	1.8	0.48
Naltrexone	1.36	9	-0.24	-1.4
Amphetamine	1.8	10	-0.7	-0.8
Methamphetamine	2.24	10	-0.5	-0.6
MDMA	1.86	10	-1.01	-0.8
MDA	1.43	10	-0.9	-1.24
MDEA	2.2	10	-0.5	-0.6
Tramadol	2.45	9	0.3	-0.6
Carisoprodol	1.92	NA	1.92	1.92
Buprenorphine	3.55	9	1.51	0.9
Norbuprenorphine	2.3	10	0.3	0.05

Table 2: Comparison of the various Log P values, ionisable pKas and Log D values and specific pH [4].

which performed adequately in the presence of acid precipitation were typically the ones with the lowest Log D values at pH 1 (Table 2). For example, amphetamine (Figure 14) shows good recovery and has a Log D at pH 1 of -0.8. The extremely high concentration of acidic salts creates a very polar solution and because the amphetamines are also relatively polar at this pH, they are relatively soluble and provide decent recovery. By contrast, EDDP exhibits poor recovery in acid (Figure 18) and displays a high Log D value at low pH implying that it is not soluble in the polar salt solution.

Organic Pretreatments

90:10 (v/v) ACN:MeOH

As seen in Figure 15, the opiates responded very well and produced the best results with an osmotic breakdown and ACN/MeOH precipitation pretreatment step. This information was used as an optimisation step in the $ZnSO_4$ extraction later described.

10:90 (v/v) ACN/MeOH

In addition, the majority of the benzodiazepines also produced good response using a combination of ACN and MeOH. However, unlike the opiates, 10:90 (v/v) ACN/MeOH produced more acceptable recovery despite the discoloured supernatant. An example is shown in Figure 17 for the chromatographic overlay of Nordiazepam.

While benzodiazepines carry relatively similar Log P values in comparison to synthetic opiates, their Log D values at pH7 are much different (Table 2) which explains why the majority of benzodiazepines looked best under this pretreatment. It is shown previously [7] that acetonitrile does a poor job extracting hydrophobic analytes in comparison to short chain alcohols. The data here supports this idea that the moderately hydrophobic benzodiazepines require a large amount of a protic solvent like methanol to achieve acceptable solubility or a zinc sulphate modifier that effectively lowers pH [5] enough to reduce the Log D to make the compound soluble in ACN. Lorazepam (Figure 21) is good evidence that $ZnSO_4$ lowers pH as it is seemingly immune to $ZnSO_4$ pretreatment since it does not contain an ionisable amine group and requires methanol to be extracted.

$ZnSO_4$ Pretreatment + ACN

The $ZnSO_4$ and acetonitrile combination yielded the best results for Benzoylecognine and Amphetamines (Figures 14 and 16). While the strictly organic pretreatment yielded slightly better recoveries for the hydrocodone and codeine, they also performed adequately with $ZnSO_4$ in place of the osmotic breakdown. A poor performer under these conditions was EDDP. A Log D value of 2.15 at pH7 implies that it was too hydrophobic to be efficiently extracted by the ACN even in the presence of $ZnSO_4$, and required the addition of 10% methanol (v/v) to achieve acceptable recovery.

$ZnSO_4$ Pretreatment + 90:10 (v/v) ACN/MeOH

Based on what was learned in the above organic pretreatments, this method was further optimised to improve the recovery of the opiates by using $ZnSO_4$ in combination with 90:10 (v/v) ACN/MeOH. Overall this pretreatment produced the most consistent results for many of the compounds in the suite (Table 3). This solvent cocktail proves to be most effective as it provides a diverse set of modifications. The $ZnSO_4$ helps lyse the cells, lowering pH and decreasing Log D values of ionisable compounds. The use of a protic solvent like methanol helps to solubilise stubborn hydrophobic compounds in the presence of the more polar protein precipitating ACN.

Other Analytes of Note:

MDMA (Figure 19) and Tramadol (Figure 20) display relatively similar responses in all of the precipitating solvents. Their polar nature at acidic and neutral pH (Table 2) support the claim that these compounds are well solubilised by any of these precipitating reagents and maybe offer a benchmark for compounds of similar chemical structure.

Conclusion:

While there is no one pretreatment option that yields the best results for each class of compounds, in this study it is shown that a very effective pretreatment using $ZnSO_4$ and 90:10 (v/v) ACN/MeOH has been developed for most compounds that are often found in a large pain panel suite representative of a typical application in forensic toxicology. By successfully preparing samples for a mixed mode cation-exchange SPE cartridge prior to LC/MS/MS analysis, column lifetime is preserved and system maintenance is abated while also eliminating other downstream chromatographic difficulties.

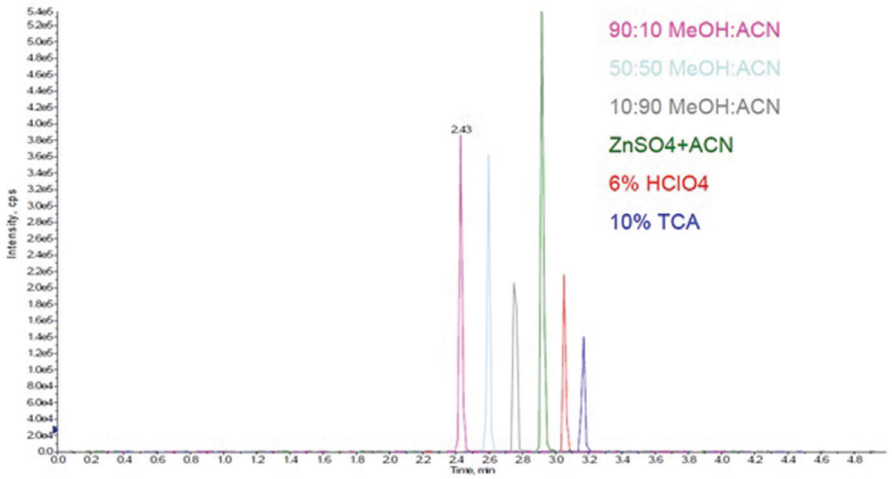

Figure 17: Comparison of the effects of various pretreatment options on Nordiazepam. Chromatograms are overlaid with time shift to provide clarity [1].

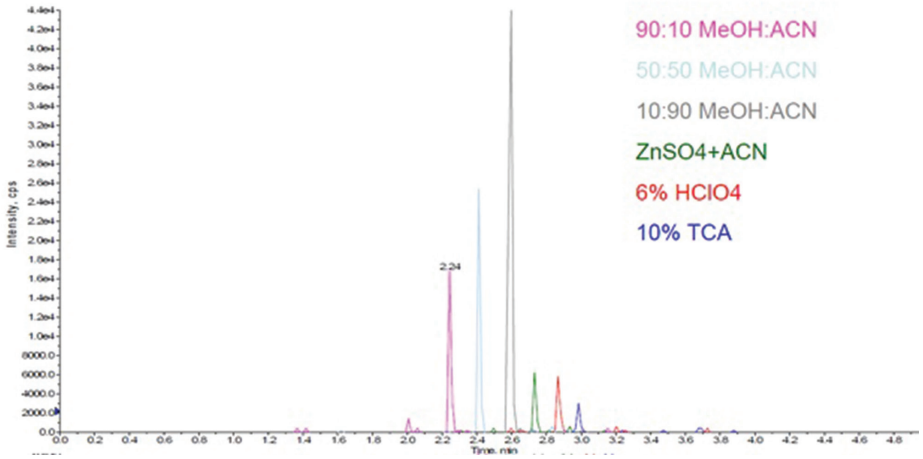

Figure 18: Comparison of the effects of various pretreatment options on EDDP. Chromatograms are overlaid with time shift to provide clarity [1].

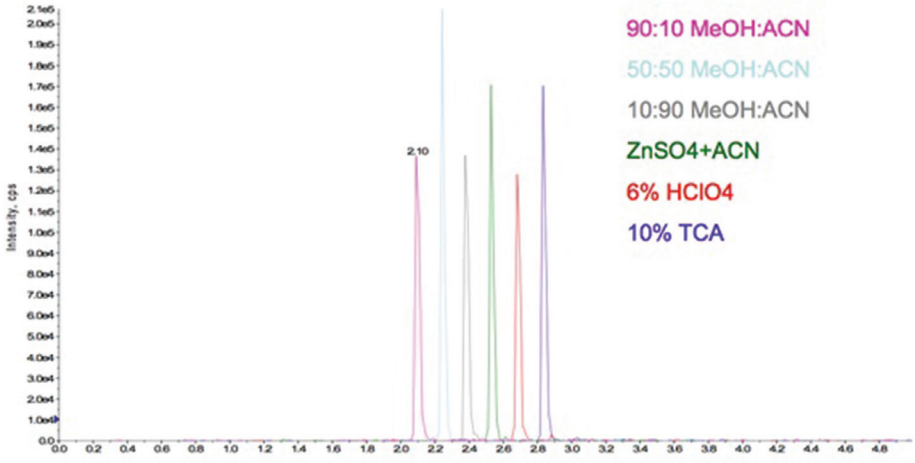

Figure 19: Comparison of the effects of various pretreatment options on MDMA. Chromatograms are overlaid with time shift to provide clarity [1].

One of the pretreatment options not investigated in this study is a technique that uses dilution in buffer followed by physically denaturing sample via sonication. The promises of such a method are elaborated on in (Chen et al., 1992) [3], where it is demonstrated that using this pretreatment for a range of acidic, basic and neutral drugs extracted on a similar mixed mode cation-exchange resin yielded the best recoveries in comparison to the other techniques

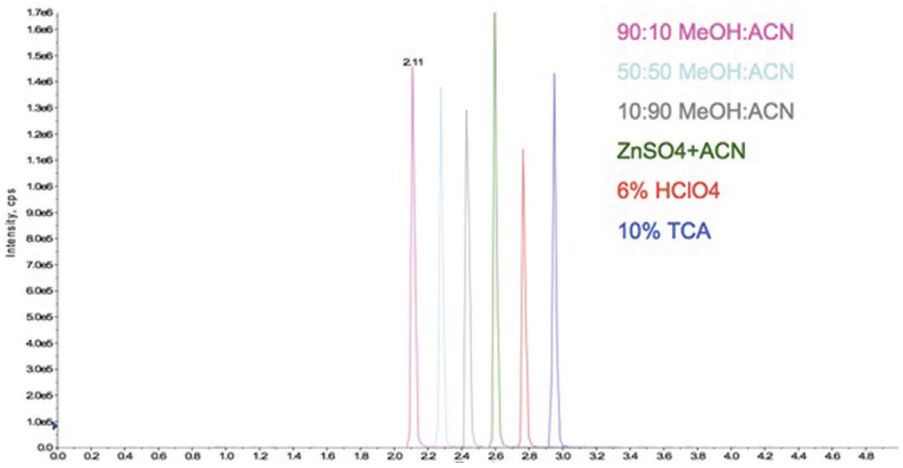

Figure 20: Comparison of the effects of various pretreatment options on Tramadol, Chromatograms are overlaid with time shift to provide clarity [1].

put forth in this study [2]. This technique does not subject the sample to protein precipitation which helps mitigate loss of analyte due to co-precipitation. This method is also enticing because it does not put forth any additives that could possibly interfere with the ion exchange mechanism (Zn^{2+}) or reduce hydrophobic retention (MeOH and ACN) [2]. However, it is shown in this work that these phenomena are at least partially mitigated by the dilution in aqueous prior to loading onto the SPE cartridge.

In summary, regardless of the sample preparation technique chosen for whole blood, it is justly important to ensure the analyte-protein interaction is completely broken and that the analyte is released and solubilised into the liquid part of the sample prior to analysis [2].

References:

1. Sadjadi, S., Huq, S., Orlowicz, S., Snow, L., Kaspick, A. "Comparison of Different Whole Blood Sample Pretreatment Methods for Targeted Analysis of Basic Drugs". MSACL 2015. Phenomenex. San Diego. April 1st 2015. Poster reference.

2. Simpson, Nigel J K. Solid-Phase Extraction: Principles, Techniques and Applications. New York: Marcel Dekker, 2000.

3. Chen, X-H., Franke, J-P., Wijsbeek J. and

Analyte	Class	Expected Conc, ng/mL (Low)	%RSD (Low)	% Accuracy (Low)	Expected Conc, ng/mL (High)	%RSD (High)	% Accuracy (High)
Alprazolam	Benzodiazepines	20	10	108	200	12	104
Clonazepam		20	9	114	200	11	107
Diazepam		20	10	97	200	12	103
Flunitrazepam		20	7	112	200	7	105
Lorazepam		20	15	108	200	10	111
Midazolam		20	7	115	200	4	88
Nordiazepam		20	11	101	200	13	103
Oxazepam		20	6	108	200	12	105
Temazepam		20	7	105	200	9	99
α-Hydroxyalprazolam		20	6	88	200	11	91
Codeine	Opiates	20	10	92	200	9	87
Oxycodone		20	4	95	200	2	93
Hydromorphone		20	6	85	200	14	97
Hydrocodone		20	7	105	200	9	99
Morphine		20	8	91	200	10	86
Methadone	Synthetic Opioids	20	10	110	200	5	105
EDDP		20	10	98	200	2	94
6-MAM		20	7	100	200	7	100
Fentanyl		20	9	115	200	5	90
Norfentanyl		20	12	95	200	4	100
Meperidine		20	7	105	200	7	103
Normeperidine		20	9	103	200	10	102
Naloxone		20	7	118	200	3	111
Norpropoxyphene		20	9	100	200	14	90
Propoxyphene		20	12	111	200	5	101
Sufentanil		20	8	98	200	7	89
Naltrexone		20	4	113	200	11	108
Amphetamine	Amphetamines	20	9	107	200	11	107
Methamphetamine		20	10	115	200	3	96
MDMA		20	13	111	200	8	92
MDA		20	8	102	200	7	101
MDEA		20	16	107	200	3	105
Tramadol	Analgesics	20	4	105	200	3	96
Carisoprodol		20	8	106	200	9	100
Buprenorphine		20	12	104	200	11	101
Norbuprenorphine		20	6	105	200	13	106
Phencyclidine	Illicit Drugs	20	7	110	200	4	92
Benzoylecgonine		20	10	104	200	5	101

Table 3: Method precision and accuracy data based on replicate quality control samples using ZnSO4 and ACN as pretreatment option [1].

deZeeuw, R.A., "Isolation of Acidic, Neutral and Basic Drugs from Whole Blood Using a Single Mixed-mode Solid-phase Extraction Column." Journal Analytical Toxicology 16 (1992): 352-355. Print

4. Chemicalize properties viewer. www.chemicalize.org February 12th, 2016

5. Polson, C., Sarkar, P., Incledon, B., Raguvaran, V., Grant, R., "Optimization of Protein Precipitation Based upon Effectiveness of Protein Removal and Ionization Effect in Liquid Chromatography – tandem Mass Spectrometry." Journal of Chromatography B .Volume 785, Issue 2, 5 March 2003, Pages 263–275

6. Debamitra, C., Parameswaran, S., Kumar Dubey, V., Patra, S., "Unraveling the Rationale behind Organic Solvent Stability of Lipases." Appl. Biochem. Biotechnol. 167 (2012): 439-461.

7. Gekko, K., Ohmae, E., Kameyama, K., Takagi, T., "Acetonitrile-protein interactions: amino acid solubility and preferential solvation." Biochimica et Biophysica Acta 1387 (1998): 195-205

8. Yin, Peiyuan., Lehmann, Rainer., Xu, Guowang. Effects of Pre-Analytical Process on Blood Samples Used in Metabolomics Studies. Analytical Bioanalytical Chemistry. 407 (2015): 4879-4892.

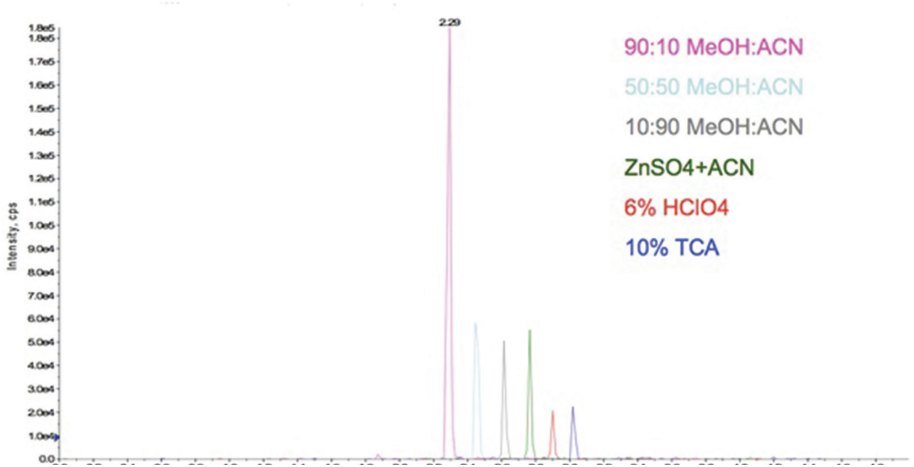

Figure 21: Comparison of the effects of various pretreatment options on Lorazepam, Chromatograms are overlaid with time shift to provide clarity [1].